

Charcoal-Yeast Extract Agar: Primary Isolation Medium for *Legionella pneumophila*

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Charcoal-yeast extract agar is a new bacteriological medium that supports excellent growth of the *Legionella pneumophila*. It results from modifications made in an existing *L. pneumophila* medium, F-G agar. Yeast extract, instead of an acid hydrolysate of casein, serves as the protein source. Beef extractives and starch are not added. Activated charcoal (Norit A or Norit SG) is included at 0.20% (wt/vol). Comparison of charcoal-yeast extract and F-G agars showed that a greater number of colony-forming units of *L. pneumophila* was recovered from a standardized tissue inoculum on charcoal-yeast extract agar (4.35×10^6 colony-forming units) than on F-G agar (4.85×10^4 colony-forming units). Macroscopic colonies of *L. pneumophila* were visible on the new medium within 3 days, whereas 4 days of growth was required on F-G agar.

McDade et al. initially isolated the Legionnaires disease bacterium, now named *Legionella pneumophila*, by using guinea pigs and embryonated chicken eggs (2, 9). For the purpose of growing *L. pneumophila* on artificial media, Weaver inoculated 17 different bacteriological agars with an *L. pneumophila*-infected yolk sac suspension. Mueller-Hinton agar supplemented with 1% hemoglobin and 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) (MH-IH) was the only medium that supported growth of this organism (5). Feeley et al. analyzed this medium and determined that L-cysteine hydrochloride and soluble ferric pyrophosphate could replace IsoVitaleX and hemoglobin, respectively, and that growth of *L. pneumophila* was best at pH 6.9 at 35°C under 2.5% CO₂. These findings were incorporated into a new medium, F-G agar. Isolation of *L. pneumophila* from a standardized guinea pig tissue proved superior on this newly formulated medium as compared with isolation on MH-IH agar (5).

This report describes modifications to F-G agar that have resulted in a greatly improved medium, charcoal-yeast extract (CYE) agar, and compares the capabilities of CYE and F-G agars to support growth of *L. pneumophila* from an infected guinea pig tissue.

MATERIALS AND METHODS

Strains. Four strains of *L. pneumophila* were used in this study. Three strains had been isolated from humans with Legionnaires disease. Two strains, Philadelphia 1 and 2, were from fatal cases of the 1976

outbreak in Philadelphia (6). The third strain, Flint 1, had been isolated from a patient with pneumonia in Flint, Mich. (4). The fourth strain, Pontiac 1, had been cultured from the lung and spleen tissues of a guinea pig exposed to the air in a building in Pontiac, Mich., the site of an outbreak of Legionnaires disease in 1968 (7). All strains had been maintained on F-G agar at 35°C and 2.5% CO₂ and transferred weekly to fresh F-G agar (5).

Medium evaluation. Each type of medium prepared was evaluated for support of *L. pneumophila* growth with both tissue and stock culture inocula. Each medium was also analyzed for both brown coloration of the medium and also examined in the dark with long-wave ultraviolet light (365 nm) for yellow fluorescence. The tissue inoculum was a suspension of *L. pneumophila*-infected guinea pig spleen that was prepared to approximate human tissue, which was not available in sufficient quantity to be used for numerous experiments. Changes in the *L. pneumophila* bacterium that could result from growth on bacteriological media were avoided by obtaining a hen egg yolk sac suspension of the Philadelphia 1 strain from Joseph E. McDade of the Center for Disease Control, Atlanta, Ga. This strain had never been exposed to bacteriological media. A 0.2% (vol/vol) suspension of this in phosphate-buffered saline, pH 7.2, was injected intraperitoneally into 15 guinea pigs (1.0 ml/animal). The animals became moribund in 4 days and were sacrificed. Their spleens were aseptically removed, emulsified to a 10% (wt/vol) suspension in phosphate-buffered saline (pH 7.2) with a mortar and pestle with 60-mesh alundum as an abrasive.

Suspensions were cultured on blood agar, and those free of bacterial contamination were pooled, thoroughly mixed, and dispensed in 1.0-ml amounts into sterile screw-capped vials. These were quick-frozen in

95% ethanol and dry ice and stored at -70°C . Direct fluorescent-antibody (DFA) examination of this standardized tissue inoculum was done to determine the number of *L. pneumophila* bacteria per gram of guinea pig tissue (3). At the time of use, each vial of standardized tissue suspension was thawed quickly and diluted in phosphate-buffered saline (pH 7.2) to give a 2.0% (vol/vol) suspension. Media were seeded routinely with 0.1 ml of this suspension, and the inoculum was spread over the entire surface of the agar with a sterile glass rod. For comparison of the sensitivity of the new CYE agar with that of F-G agar, the standard tissue inoculum was seeded onto both media at dilutions in phosphate-buffered saline (pH 7.2) of 1:10, 1:100, 1:500, and 1:5,000. Five plates of each medium were inoculated per dilution and incubated both in air and in air supplemented with 2.5% CO_2 . Colonies were counted at 7 days of incubation.

Standardized stock culture inocula were prepared from strains of *L. pneumophila* maintained on F-G agar. Growth from a 4- to 5-day culture was emulsified in phosphate-buffered saline (pH 7.2) to an approximate opacity of a McFarland no. 2 standard (8). The number of *L. pneumophila* bacteria per milliliter was determined by both culture plate count determination and DFA for a standardized suspension of Philadelphia 1 (3). A bacteriological loop (4 mm in diameter) was used to seed the standardized inoculum onto plates which were streaked for isolated colonies. Media were inoculated at 35°C in air and 2.5% CO_2 unless otherwise indicated. Growth was rated at 5 days of incubation on a scale of 1+ to 4+. A score of 1+ indicated poor growth with no isolated colonies; 2+ indicated moderate growth and no isolated colonies; 3+ indicated good growth with isolated colonies; and 4+ indicated heavy growth with numerous isolated colonies.

Medium preparation. F-G agar was made as previously reported (5). CYE agar was made by substitution of yeast extract for an acid hydrolysate of casein; it contained no beef extracts or starch, but contained activated charcoal. The composition is as follows: yeast extract, 10.0 g; activated charcoal (Norit A or SG), 2.0 g; L-cysteine $\text{HCl}\cdot\text{H}_2\text{O}$, 0.4 g; ferric pyrophosphate, soluble, 0.25 g; agar, 17.0 g; and distilled water, 980 ml.

As with F-G agar, all the ingredients of CYE agar except for L-cysteine hydrochloride and soluble ferric pyrophosphate were added to 980 ml of distilled water, dissolved by boiling, and autoclaved at 121°C for 15 min and then cooled to 50°C in a water bath. Fresh solutions of L-cysteine hydrochloride (0.40 g in 10 ml of distilled water) and soluble ferric pyrophosphate (0.25 g in 10 ml of distilled water) were prepared and 0.45- μm membrane filter-sterilized separately before adding to the sterile medium base. The medium was adjusted to pH 6.9 while at 50°C by adding 4 to 4.5 ml of 1 N KOH; the medium was swirled between the pouring of each plate to keep charcoal particles suspended and poured in 20-ml quantities into sterile petri dishes (15 by 100 mm). After cooling, the medium in one plate was checked for the required final pH of 6.9 with either a surface electrode or a combination electrode in emulsified agar in distilled water.

CYE agar was developed through the following

series of experiments. All media in these studies as well as for CYE agar used Difco agar lot no. 589190 at 17 g/liter.

Yeast extract experiment. Previous studies showed that yeast extract supplementation enhanced growth of *L. pneumophila* (5). To determine whether yeast extract could replace the acid hydrolysate of casein and beef extractives in F-G agar, a medium was prepared in which Difco yeast extract (lot no. 649736) at 10 g/liter replaced the peptone components of F-G agar. In addition, NaCl was added at 0.5% (wt/vol) because yeast extract, unlike the acid hydrolysate of casein, did not contain a high content of NaCl. Ferric pyrophosphate and L-cysteine hydrochloride were added, respectively, at 250 and 400 mg/liter of medium. The final pH of the medium was adjusted to 6.90 ± 0.05 . This medium, yeast extract (YE) agar, served as a basal medium for the following nutritional experiments.

Starch experiment. MH-1H and F-G agars contain starch. Its exact role in these media is not known. To determine whether such starch addition to YE agar would be beneficial, different brands of starch (BBL Microbiology Systems, Difco, and Fisher) were incorporated at 0.15, 0.50, and 1.0%.

Charcoal experiments. To determine whether activated charcoal could enhance growth of *L. pneumophila* from infected guinea pig tissue, Norit A (Matheson, Coleman, & Bell) and Norit SG (Sigma Chemical Co., St. Louis, Mo., catalog no. C5510) were added to YE agar to give the following concentrations (in percent, wt/vol): 0.00, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.50, 0.75, and 1.00. Subsequently, YE agar containing these charcoals at 0.15% (wt/vol) was tested for the need of NaCl supplementation at concentrations (percent, wt/vol) of 0.00, 0.13, 0.25, 0.50, 0.75, and 1.00. Finally, the optimum concentration of activated charcoal in YE agar having no added NaCl was determined. Norit A and Norit SG were tested at the following concentrations (grams per liter): 0.00, 0.25, 0.50, 0.75, 1.5, 2.0, 4.0, and 6.0.

Plates were inoculated with 0.1 ml of a 1:5,000 dilution of the standardized guinea pig spleen tissue per plate. Plates were then incubated at 35°C in air with and without 2.5% CO_2 added. Plates that were incubated in air only were protected from dehydration by placing them in plastic bags. Colonies of *L. pneumophila* were counted at 3, 4, 7, and 10 days of incubation.

RESULTS

DFA examination of the standardized inocula for *L. pneumophila* bacteria showed that the stock culture inoculum contained 7.26×10^8 *L. pneumophila* per ml and the tissue inoculum contained 1.65×10^8 *L. pneumophila* per ml (Table 1).

In the medium evaluation studies, quantitation of the stock culture inoculum by bacteriological culture showed that both F-G and CYE agars supported nearly the same numbers of *L. pneumophila* colonies (4.92×10^8 and 5.02×10^8 , respectively). These were slightly less than those

detected by DFA (Table 1). However, this was not true for the standardized tissue inoculum. Whereas 1.65×10^8 *L. pneumophila* bacteria per ml were detected by DFA, F-G and CYE agars supported only 4.85×10^4 and 4.35×10^6 colony-forming units of *L. pneumophila* per ml, respectively. In addition, the growth of *L. pneumophila* from the tissue inoculum was not linearly reduced when it was serially diluted and seeded on F-G agar. More *L. pneumophila* colonies (97) grew on F-G agar from the 1:500 dilution than from the 1:10 and 1:100 (8 and 13 colonies, respectively). Inocula of the 1:5,000 and 1:50,000 dilutions produced 6 and 0 colonies, respectively. In contrast, CYE agar seeded with similar inocula supported colonies too numerous to count from all dilutions except the highest (1:50,000), in which it supported 87 *L. pneumophila* colonies.

L. pneumophila colonies from the stock culture suspension were visible at 2 days on both media as compared with *L. pneumophila* colonies from the tissue suspension which appeared usually at 3 days for CYE agar and 4 days for F-G agar. The colonial characteristics of the *L.*

pneumophila were not the same on CYE agar as on F-G agar. *L. pneumophila* colonies on CYE agar were smoother and lacked the cut-glass texture when observed microscopically with the light illumination from one side at a 10° angle. No browning of the media was noted, and no yellow fluorescence was detected when plates were examined in the dark with 365-nm ultra-violet light.

In the series of experiments that formulated CYE agar, all four strains of *L. pneumophila* grew well (3+ or 4+) from the standardized stock culture inoculum on YE agar with or without added starch. However, only a few *L. pneumophila* colonies developed on these media from the tissue inoculum, and this was only on media containing 1% starch. However, *L. pneumophila* colonies grew well from both standard inocula on YE agar supplemented with charcoal. *L. pneumophila* colonies appeared within 2 days from the stock culture inoculum and within 3 days usually from the tissue inoculum on YE agar containing $\geq 0.10\%$ activated charcoal. Colonies were initially 1 to 2 mm in diameter and had a slightly cut-glass appearance. On continued incubation, they increased in diameter and became smooth in texture. No browning of the medium or yellow fluorescence was observed.

When NaCl was not added to YE agar supplemented with 0.15% Norit A, larger number of *L. pneumophila* colonies were detected in the tissue inoculum, but not in the stock culture inoculum. Subsequently, in the experiment designed for determining the optimum concentrations of activated charcoal in YE agar without added NaCl, 0.15% Norit A and 0.20% Norit SG were found to give good growth of the *L. pneumophila* from both of the standardized inocula (Table 2).

TABLE 1. Results of quantitating the standardized inocula for *L. pneumophila* by DFA and by bacteriological culture on F-G and CYE agars

Inoculum type	Method of quantitation		
	DFA ^a	F-G agar ^b	CYE agar ^b
Stock culture	7.26×10^8	4.92×10^4	5.02×10^8
Tissue	1.65×10^8	4.85×10^4	4.35×10^6

^a Number of *L. pneumophila* per milliliter of standardized inocula.

^b Number of colony-forming units per milliliter of standardized inocula that developed.

TABLE 2. Effect of charcoal incorporation in YE agar on the growth of *L. pneumophila* from infected guinea pig spleen tissue

Charcoal concn (g/liter)	Growth of <i>L. pneumophila</i> on media containing:															
	Norit A charcoal ^a								Norit SG charcoal ^a							
	Air				Air + 2.5% CO ₂				Air				Air + 2.5% CO ₂			
	Day 3	Day 4	Day 5	Day 7	Day 3	Day 4	Day 5	Day 7	Day 3	Day 4	Day 5	Day 7	Day 3	Day 4	Day 5	Day 7
0.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.25	0	0	5	16	0	0	0	0	0	0	1	2	0	0	0	0
0.50	0	8	29	51	0	0	1	4	0	0	8	40	0	0	0	1
0.75	0	28	53	63	0	0	6	52	0	20	52	77	0	1	4	34
1.50	14	50	70	77	0	25	59	87	0	17	46	85	0	1	23	85
2.00	18	48	69	74	2	43	74	91	10	50	71	79	0	25	63	89
4.00	24	56	72	75	20	67	88	91	16	56	72	74	13	68	96	103
6.00	17	50	69	72	39	73	92	93	29	68	84	86	6	63	89	98

^a Values represent colony counts of *L. pneumophila* on media not supplemented with NaCl at various days of incubation and are the average of five plates rounded to the nearest whole number. The inoculum was 0.1 ml per plate of a 1:5,000 dilution of the standardized guinea pig spleen tissue suspension.

L. pneumophila colonies appeared earlier on plates incubated in air than on plates incubated in air and 2.5% CO₂. However, a slightly greater number of *L. pneumophila* colonies developed on plates in CO₂-supplemented air.

DISCUSSION

Legionnaires disease is an acute bacterial disease that is usually manifested as a pneumonia. Since Legionnaires disease can be life-threatening, it should be promptly diagnosed. For this purpose, direct immunofluorescent reagents have been developed (3). The criteria of Cherry et al. for reporting results of the direct immunofluorescent tests as positive for *L. pneumophila* are that there are >5 strongly fluorescing bacteria per smear of pleural fluid and >25 strongly fluorescing bacteria per smear of lung tissue, which includes scrapings of Formalin-fixed tissues, fresh tissue imprints, and homogenates and histological sections (3). Since smears contain ~1 µl of fluid, an inoculum of 50 µl seeded on a CYE agar plate should contain ~250 and ~1,250 *L. pneumophila* bacteria, respectively, for these tissues. Our findings indicate that CYE agar should support growth of *L. pneumophila* from inocula having these concentrations of *L. pneumophila* bacteria, that is, provided that the *L. pneumophila* bacteria are viable and that the tissue is not heavily contaminated or contains a high concentration of antibiotics. Evidence for this is that CYE and F-G agars detected approximately the same number of *L. pneumophila* bacteria in the stock culture inoculum as were detected by DFA staining (Table 1). This indicates that both F-G and CYE agars are capable of supporting growth of individual cells of *L. pneumophila* that have been exposed to bacteriological media. However, ~10² *L. pneumophila* bacteria appear to be required for CYE agar to support *L. pneumophila* growth from tissue that has been frozen. Although we believe that the sensitivity of CYE agar is greater than this, CYE agar should support growth of *L. pneumophila* from DFA-positive lung tissue and pleural fluid having ≥100 bacteria per 50 µl.

Two possible explanations for CYE agar detecting fewer *L. pneumophila* bacteria than DFA staining are (i) that one *L. pneumophila* colony may develop from several *L. pneumophila* bacteria and (ii) many *L. pneumophila* cells may have become nonviable as a result of the grinding of the spleen tissue and the subsequent freezing and thawing of it.

However, these reasons do not explain why F-G agar supported ~10² fewer *L. pneumophila* bacteria from the tissue inoculum than CYE

agar. We believe the ultimate reason for this difference is that there is a physiological difference between *L. pneumophila* bacteria grown in tissue and *L. pneumophila* bacteria grown on bacteriological media. Some evidence for this is the fact that stock culture inocula grew luxuriantly on YE agar, but only a few *L. pneumophila* cells grew from the tissue inoculum on YE agar not supplemented with activated charcoal. Further support is that ~10² more *L. pneumophila* colonies developed from the tissue inoculum on YE agar supplemented with activated charcoal and having no added NaCl. This was not true for stock culture inocula. This information suggests that the difference in charcoal and NaCl content of the two media may be the immediate reason for the difference in sensitivity of the two media. The fact that F-G agar containing 0.20% Norit SG and dialyzed Mueller-Hinton base (NaCl removed) supported approximately the same number of *L. pneumophila* from the tissue inoculum as CYE agar substantiates this (J. C. Feeley, R. J. Gibson, and G. W. Gorman, unpublished data).

The inhibition of *L. pneumophila* growth by tissue debris is probably the cause of fewer *L. pneumophila* colonies developing on F-G agar from lower dilutions than from higher dilutions of the standardized tissue inoculum. Low dilutions containing considerable amount of tissue debris should be more inhibitory to the growth of *L. pneumophila* bacteria than higher dilutions of the inoculum that contain little or no tissue debris.

The absence of inhibition of *L. pneumophila* growth on CYE agar seeded with similar inocula may be the result of this medium containing activated charcoal and having no added NaCl. The charcoal may be acting as a detoxifier. However, it may also act as a collector of CO₂ and a surface tension modifier. We do not know why NaCl deletion from charcoal-containing media enhances growth of the *L. pneumophila*.

With the development of CYE agar, the incubation time required for growing *L. pneumophila* bacteria from tissue is shortened by 1 day. Quicker isolations of *L. pneumophila* bacteria should result from clinical as well as environmental specimens, especially for those in which guinea pigs are used in the isolation procedure (10).

Although CYE agar is more sensitive than F-G agar, it lacks the helpful diagnostic features that the F-G agar has. *L. pneumophila* colonies on CYE agar do not have the cut-glass appearance and do not produce the brown pigmentation of the media as seen with F-G agar. Addition of tyrosine to CYE agar to levels found in F-G

agar may correct the lack of browning (1).

We recommend that both CYE and F-G agars be used in the processing of clinical specimens—CYE agar for its sensitivity and F-G agar for its diagnostic characteristics. Whereas CYE agar can be incubated aerobically, F-G agar must be incubated in air and 2.5% CO₂. However, both media should not be incubated in higher concentrations of CO₂ because *L. pneumophila* growth may be inhibited by the pH of the medium becoming too acidic.

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